Loss of Keratin Expression in Anaplastic Carcinoma Cells Due to Posttranscriptional Down-Regulation Acting in trans


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ABSTRACT

Rat keratin K5 and vimentin complementary DNAs have been isolated, identified, and used to study keratin and vimentin expression as markers for cell differentiation. Isologous rat neoplastic epithelial cell lines used were based on a clonal benign epithelial line (A5P/B10) and a clonal anaplastic malignant derivative line (T952/F7). Stable cytoplasmic miRNA was detected for keratin but not vimentin in the benign cells. The anaplastic derivative cells expressed vimentin but showed a 1000-fold reduction in the keratin message, which nuclear run-on assays identified as being due to posttranscriptional down-regulation. An identical pattern of posttranscriptional down-regulation was found in independent malignant somatic cell hybrids of the benign and anaplastic cells. trans-acting regulatory mechanisms implicated in posttranscriptional (pretranslational) keratin down-regulation in these anaplastic malignant cells may play a role in the apparent loss of differentiation evident in tumor progression.

INTRODUCTION

Loss of cellular differentiation is a feature of progression toward a more anaplastic, metastatic, and malignant phenotype in many tumors (1). In high-grade carcinomas the presence of “sarcomatous” cells apparently arising from epithelial cells appears to be paradoxical in the context of current concepts of tissue differentiation. Characteristically, the cells of high-grade, anaplastic, “spindle-cell” carcinomas exhibit many features of mesenchymal differentiation. The anaplastic cells have an elongated, fibroblastoid shape, rather than the symmetrical cobblestone shape typical of epithelial cells, and they can express vimentin intermediate filaments, together with a partial or total loss of differentiation markers such as desmosomes and keratin intermediate filaments (2, 3). The genetic events underlying this extreme change in phenotype remain undefined. The opportunity to study the genetic mechanism responsible for the change in intermediate filament expression in this defined experimental system was therefore undertaken. The Type II keratin K5 was selected as most likely to provide an insight into the mechanism responsible for the failure of keratin expression in the anaplastic cells. Keratin K5, together with its Type I partner K14, is present in a wide range of epithelial tissues derived from ectoderm (7) and is expressed by viable human epidermal cells regardless of their level of stratification (8). Furthermore, K5 expression has been shown to be an important marker capable of distinguishing between normal and tumorigenic epithelial cells from human breast (9). The expression of vimentin, one of the Type III class of intermediate filament proteins found in “mesenchymal” cells in normal tissues and in many cultured cells (10), was also investigated in addition to keratin K5, since vimentin is frequently demonstrated in the fibroblastic cells of spindle cell carcinomas.

This paper presents evidence that the absence of keratin in experimental anaplastic carcinoma cells is due to the down-regulation of keratin expression that occurs as the result of a mechanism operating at a posttranscriptional level and corresponds with a transcriptional up-regulation of vimentin. It is proposed that a mechanism of the type described for keratin down-regulation could contribute to the apparent loss of tissue-specific differentiation markers observed in anaplastic carcinomas.

MATERIALS AND METHODS

Cell Lines. All the cell lines used in this study, apart from the leukemic cells, were derived from the same inbred strain of rats. The benign clonal epithelial cell line A5P/B10, and the two cell lines derived following cotransfection of A5P/B10, the benign cell line Y43BP and the anaplastic clonal cell line T952/F7, have been described previously (4). Briefly, the A5P/B10 cell line was cloned from a spontaneous low-grade paraaerial squamous cell carcinoma. The anaplastic T952/F7 cell line was cloned from a lymph node metastasis in an animal inoculated with A5P/B10 cells cotransfected with pSV2neo (11) plus genomic DNA from a cell line (BC1) derived from a spontaneous metastatic mammary carcinoma (12). The benign keratin-positive Y43BP cell line was obtained from a benign tumor produced by the inoculation of A5P/B10 cells cotransfected with pSV2neo and genomic DNA from normal isologous lymphocytes. Expression of the neo gene was confirmed by Northern analysis with a 32P-labeled oligonucleotide specific to neomycin.

The A5P/B10 cell line was maintained in Joklic’s medium with 10% calf serum, the T952/F7 cell line in 50/50 Joklic’s/Ham’s F12 with 10% calf serum, and the Y43BP in the same medium with 100 mg/ml Geneticin (effective concentration). All cell lines were cultured in 5% CO2/10% O2/85% N2 in a humidified incubator at 37°C. Cell lines were subcultured by stripping with 0.1% trypsin/0.01% DNAse at 37°C. Cell lines were subcultured by stripping with 0.1% trypsin/0.01% DNAse in DBA3 when cultures were approximately 95% confluent. Cultures were not maintained beyond 10 subcultures before being replaced from frozen.
stocks. All of the cell lines were isologous, having been derived from either initial or transplanted tumors from the same inbred strain of rat.

Cells from the anaplastic clonal line T952/F7 were transfected with the pSV2gpt plasmid (13) without carrier DNA by the same method as previously used (4). Following transfection the cells were selected in Joklic's/F12/10% calf serum medium containing methotrexate, hypoxanthine, thymidine, mycophenolic acid, and xanthine as previously described (13). Selection was maintained continuously, and the resistant colonies were allowed to grow together. The malignant properties of the transfected cells were confirmed by inoculation into the footpads of syngeneic animals. Retention of the transfected gene was confirmed by Northern blotting of total RNA extracted with a 32P-labeled oligonucleotide specific to guanine phosphoribosyl transferase. The transfected cell lines were designated T952/F7gpt.

Cell lines were confirmed to be free of Mycoplasma infection by the Gen-Probe (San Diego, CA) test. Procedures used to check the behavior of the cells in vivo were approved by the Animal Ethics Review Committee of the University of Sydney.

Somatic Cell Hybrids of Benign and Anaplastic Cells. Somatic cell hybridization between the benign and anaplastic cells was carried out by exposing cocultures of equal platings (2 x 10^6/90-mm dish) of the benign Y43BP (neo) and anaplastic T952/F7gpt to 30% polyethylene glycol (PEG-1500) for 30–40 s, washing, then culturing in nonselection medium (Joklic's/F12/10% calf serum). Twenty-four h later the cultures were incubated in double selection medium containing a mixture of the two selective agents Geneticin and methotrexate, hypoxanthine, thymidine, mycophenolic acid, and xanthine. The sensitivity of each of the parental cell lines was checked by plating the cells separately into double selection medium. Selection was maintained until a sufficient number of cells were available for inoculation into the footpads of syngeneic animals (21–28 days). The initial hybrids were designated F4TQ2A.

Primary cultures were prepared directly into double selection medium from a primary footpad tumor (designated F4TQ2AF) and its lymph node metastasis (designated F4TQ2AL). Each of these cell lines was then diluted cloned in double selection medium, and 12 clones, 6 from the primary neoplasm (designated as F4TQ2AF with suffixes for each clone, /A1, /A5, /B4, /B6, /C1, /C5) and 6 from the lymph node metastasis (designated as F4TQ2AL with suffixes for each clone, /D4, /D11, /F8, /G2, /G3, /G7), were selected randomly for further analysis.

Flow Cytometry. The benign (Y43BP) and anaplastic (T952/F7gpt) cells used as parent cells to produce the hybrids were cultured to near-confluence and then enzyme-harvested, fixed in ice-cold absolute methanol, washed 3 times in DBA, and then incubated on ice for 30 min in 100 µl of antibody (DAKO polyclonal rabbit anti-bovine muscle diluted 1:10 in DBA plus 1% fetal calf serum plus 0.02% azide). As a control for nonspecific staining the same procedure was used with nonimmune rabbit serum. The cells were washed in DBA plus 1% fetal calf serum for 10 min on ice to remove unbound antibody and then stained by adding 100 µl of fluorescein isothiocyanate-conjugated secondary antibody (goat anti-rabbit Ig-fluorescein isothiocyanate; Pierce). The stained cells were washed, filtered through 80-µm nylon, gauze, verified microscopically as single cells, and analyzed with a Becton-Dickinson FACScan flow cytometer using Lysis II software collecting 10,000 events at a low flow rate (200–300 events/s).

Isolation of Rat Keratin and Vimentin cDNAs. A cDNA library produced from isologous rat BCl cells (12) was prepared from oligodeoxythymidine-purified cytoplasmic mRNA and cloned into the EcoRI site of Agt10 by standard methods (14). Approximately 3 x 10^6 plaques were screened by hybridizing with a 32P-labeled sheep keratin K5 cDNA (kindly donated by Professor George Rogers, Department of Biochemistry, University of Adelaide, South Australia, Australia).

Of the 3 x 10^6 plaques screened, 30 positives from the primary and 20 positives from secondary screening were selected. Polymerase chain reaction of those 20 clones was performed (15) using the 21 mer and 24 mer Agt10 sequencing primers 1231 and 1232 (New England Biolaboratories). Four clones were identified to be larger than 1000 base pairs. Multiprime 32P-labeled polymerase chain reaction DNA of the 4 clones was used to probe Northern blots, and 2 unique transcripts were identified. Lambda phages containing these 2 clones were amplified, and the cDNAs were excised from the EcoRI cloning site. cDNAs were collected from a low-melting-point agarose gel (1.5%) and purified using a NACS PREPAC convertible column (Bethesda Research Laboratories, Cat. no. 1525NP).

Both of these cDNAs were subcloned into pGem-7zf(+) at the EcoRI cloning site, and initial sequencing from the extremes of each transcript with T7 DNA polymerase was performed on alkali-denatured plasmid DNA using the dideoxy chain termination method (16). cDNA identity was confirmed by a comparison of single-strand data from each extreme of both inserts with the GenBank data base.

Preparation and Analysis of RNA. For Northern blotting, 2 µg (unless otherwise stated) of total cytoplasmic RNA (14) were electrophoresed on 1% agarose gels containing 2.2 mM formaldehyde in running buffer (17) and transferred by capillary blotting to GeneScreen Plus.

Preparation and Analysis of DNA. For Southern blot analysis total genomic DNA was isolated (18), blotted, and hybridized (19) using standard methods. Filters were autoradiographed using Kodak XAR-5 X-ray film and an intensifying screen at ~70°C.

Nuclear Run-on Transcription. DNA slot blots for run-on analysis were prepared by blotting 4 µg of each plasmid onto GeneScreen Plus membrane. Approximately 10^6 cells were grown to subconfluence, at which time both cytoplasmic RNA and cell nuclei were isolated. Nuclei were isolated from cell lines (20) and stored at ~70°C in 400 µl of nuclei storage buffer (40% glycerol; 5 mM MgCl2; 50 mM tris-HCl, pH 8.0; and 1 mM EDTA). Transcripts were elongated in vitro, purified (21), and used to hybridize against slot-blot-prepared DNA.

Inhibitor Studies. Actinomycin D in a crystalline state (Boehringer Mannheim catalogue no. 102 008) was dissolved in ethanol, diluted in culture medium to a final concentration of 10 µg/ml, and added to the cells for specified times. Cycloheximide (Sigma Chemical Company catalogue no C 6255) was added to the total medium at a final concentration of 10 µg/ml, and the cells were cultured for the specified times. To determine the effectiveness of the drugs in the A5P/B10 and T952/F7 cell lines, equal cell numbers were plated into 96-well culture plates (approximately 2 x 10^4 cells/well) using 90 µl of the appropriate medium, and left for 4 h to ensure cell adhesion. For Actinomycin D the drug was used in concentrations of 5 and 10 µg/ml to each cell line and left for 15 min, after which 2 µCi of [5,6-3H]Juridine was added to treated and control (untreated) wells. For cycloheximide, the drug was added at a concentration of 10 µg/ml simultaneously to the addition of 1 µCi of [4,5-3H]Juridine after prior leucine starvation for 2 h. For each of the drug treatments additional wells acted as blanks for background radioactivity. The plates were incubated at 37°C for 3 h. Wells were washed 2 x with phosphate-buffered saline and cells were dislodged with 1% trypsin and collected on GF/C filters (Whatman, Ltd., Kent, England) to a specific activity of approximately 2 x 10^6 cpm/µg. Oligomers were end-labeled using [α-32P]ATP (22).

Synthetic Oligonucleotides. Oligonucleotides for internal coding regions of neomycin and xanthine guanine phosphoribosyl transferase (neo) 5'-TAT GTC CTG ATA GCG GTC CGC C-3' and gpt 5'-CAA CCA GGC GAC GAC CAG CCC G-3' were prepared on an Applied Biosystems DNA synthesizer based on sequence information from the GenBank database (accession nos. V00618 and K01784).

Probes. Double-stranded DNA transcripts were labeled with [α-32P]-dCTP using a Multiprime labeling kit (Amersham, Little Chalfont, England) to a specific activity of approximately 2 x 10^6 cpm/µg. Oligomers were end-labeled using [γ-32P]ATP (22).

RESULTS

Keratin Proteins Expressed in Benign but not Anaplastic Cells. Flow cytometry of benign and anaplastic cell lines stained with a fluorescein isothiocyanate-labeled polyclonal antibody which stained normal rat epithelial cells and the benign cells in vivo (4) was used to detect the synthesis of keratin proteins in the cells in culture. A signal an order of magnitude
above the signal for the nonimmune serum control was evident in the benign transfected Y43BP cells (Fig. 1A), while in the anaplastic transfected T952/F7gpt cells the signal was not above the background level (Fig. 1B).

Species-specific Probes for Intermediate Filament Transcripts of Keratin and Vimentin. Screening of a rat cDNA library resulted in the subcloning of cDNAs for rat keratin K5 and vimentin. These clones are referred to as pRK5 for rat keratin K5 (approximately 1100 base pairs; 3' region) and pRV for rat vimentin (approximately 1400 base pairs; 3' region). Comparison with human keratin K5 (23) showed that the rat keratin K5 clone spanned a distance from exon 5 to a putative polyadenylation signal. Keratin K5 and K6 share extensive sequence homology. The possibility that the keratin cDNA coded for a K6 keratin was excluded by reading from the 3' region for 491 nucleotides which included the complete untranslated region. A similar comparison between the partial sequence data for rat vimentin (226 nucleotides into the 5' region) and Syrian hamster vimentin (24, 25) demonstrated that the rat vimentin cDNA began within exon 1 and extended to include a putative polyadenylation initiation signal. Neither transcript included any part of the polyadenylated tail.

Reciprocal Constitutive Expression of Keratin K5 in the Benign Cells and Vimentin in the Anaplastic Cells. The differential keratin expression observed in flow cytometric studies (Fig. 1) was confirmed with the rat-specific 32P-labeled pRK5 on Northern blots of total cytoplasmic mRNA from the benign (A5P/B10) and anaplastic (T952/F7) cells at equivalent gel loadings (10 μg) (Fig. 2). At longer exposure times a definite signal for the keratin message was evident in the A5P/B10 cells at a loading of 100 ng, while in lanes loaded with 10 μg T952/F7 cytoplasmic RNA the signal could not be distinguished from background. Keratin expression for T952/F7 cells was estimated as 1000-fold less than for A5P/B10 cells.

Reciprocal pattern of expression of vimentin versus keratin was found when cytoplasmic RNA from the two cell lines was probed with the rat-specific pRV probe, irrespective of the number of times the cells were passaged (Fig. 3). Signals were assessed relative to the expression of the two housekeeping genes GAPDH (American Type Culture Collection catalogue no. 57090) and a rat ribosomal protein cDNA (a 534-base pair cDNA isolated from the rat BC1 cDNA library and identified by sequence comparison to GenBank accession no. X51538).
Fig. 3. Reciprocal pattern of cytoplasmic RNA expression of vimentin versus keratin for A5P/B10 and T952/F7 cells on the same membrane probed with $^{32}$P-labeled pRK5 and pRV and dominant expression of vimentin for two hybrid clones (F4TQ2AF/A1 and F4TQ2AL/D4). The expression profiles for the two labels were consistent in RNA extracted from different platings and passages of the cells.

The Keratin K5 Gene Is Retained and Not Rearranged in the Anaplastic Cells. DNA from A5P/B10 and T952/F7 cells was digested with eight restriction enzymes and subjected to Southern hybridization analysis with the pRK5 cDNA probe. The same banding patterns were evident in DNA from the T952/F7 and the A5P/B10 cells, indicating that the differences in expression of keratin K5 mRNA were not due to gross chromosomal gene rearrangements (Fig. 4A). Reprobing the Southern blots with pRV showed no alteration in the banding patterns for vimentin (Fig. 4B).

The Keratin Message Is Stable for a Minimum of 6 h in the Benign Cells. The stability of the keratin message was monitored following the addition of actinomycin D to subconfluent A5P/B10 cells at a concentration of 10 µg/ml to inhibit RNA synthesis. Cytoplasmic keratin mRNA expression was monitored over the subsequent 20-h period relative to the expression of c-myc (American Type Culture Collection catalogue no. 41029) and GAPDH mRNAs (Fig. 5A). c-myc has an unstable transcript with a half-life of less than 60 min (26-28) and was used as a control to assess the effectiveness of actinomycin D as an inhibitor of transcription. A reduction of c-myc signal could be detected by 1 h, but the signal for GAPDH, which has a half-life of several hours (28), remained constant over 6.75 h. No significant alteration of keratin mRNA levels was evident up to 6.75 h of exposure to actinomycin D (Fig. 5B), despite a greater than 95% block in the synthesis of RNA in the A5P/B10 cells as assessed by tritiated uridine uptake (Table 1). Reduction of both the GAPDH and keratin was evident at 20 h, at which time the morphology of the cells indicated that prolonged incubation in the drug had compromised cell viability. These results indicated that the keratin message was stable over several hours.

Cycloheximide Has No Effect on Keratin Expression in the Anaplastic Cells. To determine if action of a short-lived protein causing rapid destruction of mRNA could be responsible for the absence of keratin message in the T952/F7 cells, they were incubated with cycloheximide at 10 µg/ml. Protein synthesis is effectively inhibited (more than 95% in A5P/B10 and T952/F7 cells) by this concentration of drug in both cell lines as assessed by its ability to block [4,5-3H]leucine uptake over a 3-h period (Table 2). c-myc mRNA levels have been shown to

![Fig. 4. Southern blot analysis for keratin and vimentin of restriction enzyme-digested genomic DNA from A5P/B10 and T952/F7 cells. Ten µg of genomic DNA from A5P/B10 cells (odd-numbered lanes) and T952/F7 cells (even-numbered lanes) were digested with AluI (Lanes 1 and 2), BglI (Lanes 3 and 4), EcoRI (Lanes 5 and 6), HindIII (Lanes 9 and 10), HinfI (Lanes 11 and 12), MspI (Lanes 13 and 14), SacI (Lanes 15 and 16) and XbaI (Lanes 17 and 18). DNA in Lanes 7 and 8 remained undigested as determined by viewing ethidium bromide-stained agarose gel prior to DNA transfer. The same membrane was probed initially with $^{32}$P-labeled pRK5 (A) and subsequently stripped and reprobed with $^{32}$P-labeled pRV (B). No differences were evident in the number or mobility of the bands in DNA from the two cell lines.](https://cancerres.aacrjournals.org/content/53/5/6606/F5.large.jpg)
rapid response in
with c-myc, GAPDH, and ribosomal protein $24$. Actinomycin D produced a
radiographic signals for A5P/B10 cells in the gels measured by laser densitometry
relative to the respective GAPDH signal at each time interval. Keratin mRNA
over the subsequent 20-h period. The same membrane was subsequently reprobed
within 60 rain.

c-myc,
c-myc
levels were not significantly decreased up to 6.75 h, while
increase in various cell lines in response to cycloheximide, in-
dicating the action of a short-lived cytoplasmic regulatory pro-
tein (21, 29, 30). c-myc and GAPDH mRNA expression pro-
files were again used as additional controls to assess the effects
of cycloheximide throughout the experiment. Keratin expres-
sion could not be induced in the T952/F7 cells by cyclohexim-
eide expression and transcription of the keratin gene in these cells relative to the
benign A5P/B10 cells (Fig. 7). Run-on analysis of nuclei ex-
tracted from the same A5P/B10 and T952/F7 cell preparations
in which cytoplasmic RNA had exhibited differential expres-
sion by Northern analysis (Fig. 3) showed equivalent levels of
transcription of the keratin gene. Transcription of GAPDH and
$\alpha$-tubulin (31) was also equivalent in the two cell lines, whereas
vimentin transcription could be detected only in the T952/F7
cells (Fig. 7). In the rat leukemia cells used as a negative control
cell, keratin and vimentin transcription were not detectable,
while GAPDH and $\gamma$-actin (32, 33) produced strong signals,
and $\alpha$-tubulin was barely detectable. These results provided
strong evidence that a mechanism was acting posttranscription-
ally to achieve the differential keratin expression patterns ob-
erved in these cell lines.

Gene Transcription and Expression Patterns of the Anaplas-
tic Parent Cells Maintained in Malignant Hybrids of the Be-

nign and Anaplastic Cells. To determine if the reduction in
keratin expression in the anaplastic cells could be restored by
complementation with genes from the keratin-expressing be-
nign cells, somatic cell hybrids were generated by fusion be-
 tween the benign and anaplastic cells. Each of the parental lines
was stably transfected with different selectable genes (either
neo or gpt), and hybridization was ensured by selection for cells
expressing both transfected genes following fusion. The Y43BP
cell line was identical to A5P/B10 in keratin and vimentin
expression (Fig. 8) and had a similar behavior in syngeneic
hosts producing benign, keratin-positive neoplasms (4). The
phenotype of the keratin and vimentin expression in the T952/
F7gpt cells was identical to that seen in T952/F7 cells (Fig. 8),
and their behavior in syngeneic animals was also similar. Fu-
nion between these transfected cell lines and selection for ex-
pression of both transfected genes produced hybrid cell lines
which exhibited a keratin-negative (by immunoperoxidase
staining; data not shown) malignant phenotype.

The levels of keratin and vimentin mRNA detected in all the
malignant hybrid lines and clones were comparable with the
anaplastic T952/F7 (and T952/F7gpt) cells rather than the be-
nign A5P/B10 (and Y43BP) unfused cells (Fig. 8). Transcrip-
tion of four of the randomly selected hybrid clones were studied
in run-on assays, and despite the paucity of keratin message
found in the keratin-expressing A5P/B10 cells and the nonex-
pression (Fig. 6B) and run-on analysis of 4 of these cloned lines produced essen-
tially identical results.

Genomic DNA from all hybrid lines isolated (6 from each of
the primary and secondary tumors) was subjected to SacI
digestion and Southern blot analysis with radiolabeled pRK5- and
pRV-derived cDNAs (results not shown). Both keratin K5 and
vimentin genes were detected in all cell lines, thereby excluding
the possibility of chromosomal loss of one or other of these
genes. Expression of one of the transfected resistance-marker genes in each of the respective unfused cells and the expression of both of these genes in the hybrid cells were confirmed when cytoplasmic mRNA from each of the lines was probed with synthetic oligonucleotides for unique sequences in either the gpt or neo genes for unique sequences in either the gpt or neo genes.

These findings indicated that posttranscriptional down-regulation of keratin expression in the malignant hybrid cells was mediated by a trans-acting mechanism derived from the anaplastic parent cell.

**DISCUSSION**

The anaplastic cells (T952/F7) derived from the benign epithelial cells (ASP/B10) showed rapid growth in vivo and elongated bipolar morphology, with an absence of desmosomes and keratin expression, but expressed vimentin, as determined by Northern analysis. Nuclear run-on analysis clearly showed this emergence of vimentin expression in the anaplastic cells to be the result of transcriptional activation. Vimentin, while normally restricted to cells of "mesenchymal" lineage, can be expressed together with cell lineage-specific keratins when the normal function and relationships of some epithelial cell types have been deranged (34–36). The almost total absence of keratin expression in the anaplastic cells that had been derived from benign cells which expressed keratin constitutively was not due to loss or obvious rearrangement of the keratin gene. Southern blotting with pRK5 of genomic DNA digested with eight restriction enzymes established that the failure to detect keratin mRNA in the cytoplasm of the anaplastic cells had a more complex basis. The same pattern of bands for the keratin gene was detected in genomic DNA from the keratin-negative anaplastic cells as in genomic DNA from the benign antecedent cells, which both transcribed and translated the gene normally. The possibility remained that the failure to detect mRNA in the cytoplasm of the anaplastic cells was due to a point mutation not detectable by Southern analysis that had inactivated the gene and prevented the transcription of normal mRNA in the anaplastic cells. The demonstration in the run-on assays that the gene was being transcribed at the same rate in the benign expressing cells, the anaplastic nonexpressing cells, and the nonexpressing hybrids nullified such an interpretation. Rather, the evidence indicated that the absence of keratin expression in the cytoplasm of the anaplastic cells was due to the loss of the ability of the cells to process and translate the gene that was still being actively transcribed. Fusion progeny, verified by the simultaneous presence and action of two different integrated sequences...
Keratin posttranscriptional regulation in \textit{trans}.

Diverse levels of regulation have been described for some of the more than 20 genetically distinct keratins (44). For several keratins, the relevant gene is transcribed, the nuclear RNA apparently processed normally, and the mRNA transported to the cytoplasm, where translation is then subject to control (23, 45–47). Down-regulation (48) and up-regulation (49) of transcription of keratin genes have been described, while the spontaneous activation of keratin genes has been observed in many nonepithelial cells maintained in culture over prolonged periods (43). Posttranscriptional (pretranslational) down-regulation of gene expression of the type demonstrated by the anaplastic T952/F7 cells has been described for a range of proteins, including collagen (50), ferritin (51), myelin basic protein (52), enzymes (53–55), receptor proteins (56), cytokines (57), protooncogenes (58), and suppressor genes (59) and recently reported for the simple epithelial keratin K18 (60). The \textit{myc} protooncogene is one member of a group of genes for which a cytoplasmic protein (adenosine-uridine binding factor) has been identified by Gillis and Malter (57) which targets adenosine-uridine-rich sequences in the 3' untranslated region and facilitates messenger degradation. Further work will be required to define the details of the mechanism down-regulating expression of the rat keratin mRNA, but the fact that rat keratin K5 cDNA has no adenosine-uridine-rich sequences in the 3' untranslated region indicates that rapid cytoplasmic destruction of K5 mRNA by adenosine-uridine binding factor probably does not occur. Some other mechanism causing rapid destruction of mRNA following its delivery into the cytoplasm cannot be ruled out on the evidence presented; however, the demonstration that cytoplasmic keratin mRNA expression could not be induced in the anaplastic T952/F7 cells following exposure to cycloheximide suggests that the nucleus is the site of the down-regulatory event.

The results presented in this paper indicate that a change in phenotype observed in anaplastic cells (the loss of keratin expression in this case) can be achieved by a mechanism other than an obvious mutation causing loss or rearrangement of the particular gene of interest. Achieving changes in patterns of gene expression through a posttranscriptional activity involving RNA processing or stability is not unique, as the above examples show. The fact that many of these examples, including

![Fig. 7. Transcriptional activities for keratin and vimentin assessed by nuclear run-on experiments in A5P/B10, T952/F7, and F4TQ2A1/D4 cells isolated at subconfluence and rat leukemia cells in primary culture. Nuclei and cytoplasm from cells of the same passage were collected separately, and cytoplasmic mRNA levels, except for leukemic cells, were assessed by Northern blot analysis (refer to Fig. 3 for direct comparison). GAPDH, \(\alpha\)-tubulin, and ribosomal protein S24 DNA were included as positive control genes, and pGEM-7z(f+) plasmid DNA was included as a negative control. Signals of equivalent strength for keratin transcription were evident for the cells with cytoplasmic message (A5P/B10) and the cells without detectable message (T952/F7 and F4TQ2A1/D4). A signal for vimentin was evident in T952/F7 and F4TQ2A1/D4 cells but not in A5P/B10 cells. No signal for keratin transcription was detected for the leukemic cells.](image)

![Fig. 8. Northern blot analyses of keratin and vimentin expression in hybrid cell lines. Northern blots were prepared in parallel with identical total cytoplasmic RNA samples and were hybridized with either \(\text{\textsuperscript{32}P}\)-labeled pRK5 or pRV cDNAs or oligonucleotides for \textit{neo} or \textit{gpt}. Ribosomal protein S24 was used as a control gene transcript. (The second keratin row had a longer period of exposure than the first.) The pattern of keratin and vimentin expression paralleled the pattern of expression of the anaplastic parent cells (T952/F7) rather than the benign parent cells (Y43BP). End-labeled oligonucleotides of \textit{neo} or \textit{gpt} hybridized only to the respective transfected cell lines and all the fused cell lines.](image)
ours, are applicable in cell models of neoplastic origin and that many involve genes which affect cell growth would suggest that continued exploration of tumorigenic cell lines for such gene activities may open a door to information on the regulation of genes with respect to such diverse activities as cell differentiation and dedifferentiation, cell transformation, and neoplastic progression. The aberrant but stable cellular phenotypes expressed in neoplastic cells presumably are a disturbed reflection of the spectrum of genetic controls available to normal cells. In the normal cell such controls may be inactivated relics of functions required in early embryogenesis, which can be reactivated inappropriately in neoplastic cells, where they can result in dramatic changes in cell behavior (36). In normal cells, transient and controlled reactivation of similar genes could play an instrumental role in facilitating immediate cellular responses to the frequent environmental changes which require a rapid but short-lived change in cell phenotype, such as in the response to wounding. Identifying and characterizing mechanisms controlling pleiotropic effects in neoplastic cells may contribute to a better understanding of mechanisms of gene control in normal cells.

Genbank Accession Numbers. The accession number for initial sequence data is M89644 for keratin K5 (3', 491 base pairs) and M84481 for vimentin (5', 226 base pairs). The accession number for ribosomal protein S24 cDNA is M89646.

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